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(58) Field of search
C3H

(54) A method capable of determining cathepsin B in the presence of other proteolytic enzymes and compounds useful therefor

(57) A method of selectively assaying for the activity of cathepsin B in mammalian fluids and tissues which may also contain trypsin and trypsin-like enzymes using novel substrates. The substrates are of the general formula: $Z - R_2 - R_1 - X$ wherein X is an indicator moiety cleavable by cathepsin B, R_1 is an amino acid group which is not positively charged, R_2 is a hydrophobic amino group, and Z is an amino blocking group.

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SPECIFICATION

A method capable of determining cathepsin B in the presence of other proteolytic enzymes, and compounds useful therefor

This invention relates to a method capable of measuring cathepsin B activity in the presence of other proteolytic enzymes such as trypsin. This invention further relates to novel substrates which are highly specific to cathepsin B. The method of this invention is particularly useful for the detection of abnormal levels of cathepsin B activity in body fluids, such as human blood, and in body tissues.

Proteinases are enzymes that digest proteins and polypeptide chains. These enzymes are highly selective to cleavage sites of the polypeptide substrates. This enzyme activity is precisely controlled in a normal biological system. A breakdown in this control can lead to serious biological consequences. Numerous disease states have been attributed to such breakdowns or disturbances in the activity of proteinases (as discussed in A. J. Barrett, ed. (1979) *Proteinases in Mammalian Cells and Tissues*, North-Holland, New York; A. J. Barrett and J. K. McDonald (1980) *Mammalian Proteases: A Glossary and Bibliography*, Vol. 1, Academic Press, New York).

Cathepsin B is a normal lysosomal cysteine (thiol) proteinase. Recently it has been shown (A. J. Barrett and J. K. McDonald, *op. cit.*; M. Sandler, ed. (1980) *Enzyme Inhibitors as Drugs*, University Park Press, Baltimore) that an apparent breakdown in the control of cathepsin B activity may lead to a variety of disease states. Several diseases associated with collagen and structural glycoprotein breakdown have been linked to increased cathepsin B activity. In particular, a correlation between cathepsin B activity and metastatic disease has been suggested by several investigations, including the elevation of cathepsin B or cathepsin B-like activity in the blood of patients with metastatic disease [as discussed in R. J. Pietras, et al. (1978) *Obstet. Gynecol. 52*; 321-327; R. J. Pietras, et al (1979) *Gynecol. Oncol. 7*; 1-17.] Accordingly, a method for the accurate and selective assay of cathepsin B activity in mammalian body fluids would be a useful diagnostic aid, such as detecting or monitoring.

The assays of various enzyme activities are routine procedures in the clinical laboratory. Typically such assays include contacting the enzyme-containing sample with a synthetic substrate (defined as that substance or compound acted upon by the enzyme) that is selectively cleaved by that enzyme into products that change colors:

$$\begin{array}{ccc} \text{substrate} & \xrightarrow{\text{enzyme}} & \text{products} \\ \text{(no color)} & & \text{(colored)} \\ & \text{or} & \\ \text{(colored)} & & \text{(no color)} \end{array}$$

Thus, substrates for cathepsin B would be peptide or peptide-like compounds that upon cleavage allow analysis of the products. Assays that have been used for cathepsin B [as discussed in A. J. Barrett, *op. cit.*; R. J. Pietras, *op. cit.*; A. J. Barrett (1980) *Biochem. J. 187*: 909-912] have included peptide substrates that incorporate one or more arginyl or lysyl amino acids attached to an enzyme-cleavable indicator group. (e.g. Bz-Arg-NA, CBZ-Lys-PNP, CBZ-Arg-Arg-MNA, CBZ-Ala-Arg-Arg-AFC, CBZ-Phe-Arg-AMC, see abbreviations below). While the selective cleavage of synthetic substrates by cathepsin B is not yet well understood, these currently used substrates, by the nature of their positively-charged groups, will also be cleavable by the trypsin-like (serine) proteases. Thus, the assays for cathepsin B using one of these lysyl or arginyl substrates in mammalian body fluids is complicated by the presence of abundant trypsin-like proteases. In particular, it has not been known how to achieve high specificity for and detection of cathepsin B activity in the presence of trypsin and trypsin-like enzymes.

Accordingly, an object of the invention is to provide substrates highly specific for cathepsin B. It is another object of the invention to selectively assay for cathepsin B activity in the presence of other proteolytic enzymes, for example, of trypsin. It is yet another object of the invention to determine cathepsin B activity in mammalian body fluids and tissues. Further objects of the invention will be apparent from the following description.

Thus viewed from one aspect the invention provides a method of selectively assaying for the activity of cathepsin B in a material to be tested which may contain trypsin and trypsin-like enzymes, the steps comprising:

(a) mixing a sample of said material with a substrate of formula I

$$Z - R_2 - R_1 - X \qquad \text{(I)}$$

(wherein:

X is an indicator moiety released by cleavage of the $R_1 - X$ bond by cathepsin B and which is detectable upon cleavage;

R_1 is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the assay;

R_2 is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the R_1 and R_2 groups) or an acid salt thereof,

said mixing being carried out in an aqueous medium having a pH within the range at which cathepsin B is active and using a quantitiy of said substrate substantially greater than that of cathepsin B and in sufficient concentration for cleaved X-groups to be detectable; and

(b) measuring the rate of cleavage of the X-group from said substrate.

Viewed from a further aspect, the invention provides a method of detecting the activity of cathepsin B in a material to be tested which may contain trypsin and trypsin-like enzymes, and which is adapted to selectively assay for cathepsin B, the steps comprising:

(a) contacting a sample of said material with a substrate of formula I

$$Z - R_2 - R_1 - X \qquad (I)$$

(wherein:

X is an indicator moiety released by cleavage of the $R_1 - X$ bond by cathepsin B and which is detectable upon cleavage;

R_1 is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the detection method;

R_2 is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the R_1 and R_2 groups) or an acid salt thereof,

said contacting being carried out under conditions at which cathepsin B is active and at concentrations at which said substrate is present in amounts substantially greater than that of cathepsin B; and

(b) determining the production of X cleaved from said substrate.

This invention provides a method for testing for cathepsin B activity in sample materials, especially mammalian body fluids or tissues, by measuring the rate at which an indicator group X is released upon cleavage of a synthetic peptide substrate highly selective for cathepsin B.

Viewed from a further aspect, the invention provides compounds of formula I

$$Z{-}R_2{-}R_1{-}X \qquad (I)$$

(wherein X is an indicator moiety cleavable from R_1;

R_1 is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group (C_α) and that is not positively charged (within the pH range of the testing conditions), preferably having a polar side chain, and preferably in which the side chain has from 2 to 8 atoms;

R_2 is a hydrophobic amino acid residue, preferably aromatic, most preferably phenylalanyl, which has the L-configuration at the carbon alpha to the carbonyl group (C_α), and preferably has a side chain of the formula $-CH_2-Y$ wherein Y is selected from lower alkyl, phenyl, substituted phenyl and indole groups; and

Z is an amino blocking group) and the salts thereof.

The following abbreviations are used herein:

Amino Acids (all assumed to be L unless otherwise specified)

Phe	-	L-phenylalanyl group
Cit	-	L-citrullyl group
Ala	-	L-alanyl group
Arg	-	arginyl group
Lys	-	lysyl group
(NO_2)Arg	-	N^{ω}-nitroarginyl group
(Ts)Arg	-	N^{ω}-tosylarginyl (or N^{ω}-p-toluene-sulfonyl arginyl) group
Trp	-	tryptophyl group
Met	-	methionyl group
Asp	-	aspartyl group
(TFA)Lys	-	N^{ϵ}-trifluoroacetyllysinyl group
(CBZ)Lys	-	N^{ϵ}-carbobenzyloxylysyl group
Val	-	valyl group
Leu	-	leucyl group
Lle	-	isoleucyl group
Nala	-	Naphthylalanyl group

Amine Blocking Groups

CBZ	-	carbobenzyloxy group
Bz	-	benzoyl group
t-BOC	-	t-butyloxycarbonyl group

Chromophores and Fluorophores

AMC	-	7-amino-4-methylcoumarin group
HMC	-	7-hydroxy-4-methylcoumarin group
AFC	-	7-amino-4-trifluoromethylcoumarin group (See International Patent Application Publication No. WO 80/02295)
AQ	-	6-aminoquinoline group
NA	-	2-naphthylamine group
MNA	-	4-methocy-2-naphthylamine group
PNP	-	para-nitrophenol group
PNA	-	para-nitroaniline group

Synthetic Reagents and Solvents

TEA	-	Triethylamine
DCC	-	N,N'-dicyclohexylcarbodiimide
DMF	-	N,N-dimethylformamide
THF	-	tetrahydrofuran
DMSO	-	dimethylsulfoxide
HOAc	-	acetic acid
HBr/HOAc	-	acetic acid saturated with HBr
Pd-PEI	-	palladium polyethyleneimine catalyst

Miscellaneous

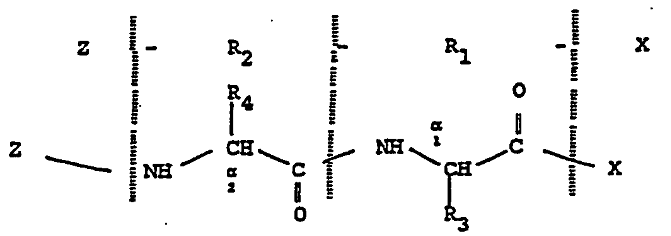
EDTA	-	ethylenediaminetetraacetic acid, disodium salt
mmole	-	millimoles
mm	-	millimeter
nm	-	nanometer
$[\alpha]_D^{25}$(DMF)	-	optical rotation at 589nm, 25°C, in DMF solvent
Bis-Tris	-	2,2-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol
HPLC	-	high performance liquid chromatography using Alltech (Deerfield, Ill.) column: 4.6x250 mm, C18; flow rate: 1 ml/min; detector: UV 340, 313, 280, and 254 nm. Mobile phase solvent, retention time, and approximate purity listed for each example.
MS	-	mass spectroscopy using a Micro Mass VG7070H instrument with fast atom bombardment probe (9KV Xenon) and sample dissolved in DMSO/glycerol.
H-NMR	-	proton nuclear magnetic resonance on a Bruker 270 MHz instrument using d_6-DMSO solvent and tetramethylsilane as an internal standard at a chemical shift (δ) of 0.0. The chemical shifts, δ, the peak splitting: s = single, d = doublet, t = triplet, q = quartet, qn = quintet, se = sextet, c = complex multiplet, and the number of protons (1p = 1 proton) are listed for each example.
MP	-	melting point (uncorrected, Fisher-Johns apparatus)

HPLC(Prep)	-	high performance liquid chromatography using Alltech (Deerfield, Ill.) column: 10x250 mm, C18, flow rate: 2 ml/min; detector: UV 340 nm. Mobile phase solvent and retention time are listed for each example.

The peptide substrate compounds are defined using the following correlation of the formula to the structure for clarity. The general formula is as follows:

$$Z - R_2 - R_1 - X \qquad \text{(I)}$$

The structural formula for the above can be written as follows:



(I)

(II)

where R_3 is the side chain for the R_1 amino acid residue, and R_4 is the side chain for the R_2 amino acid residue,

X is a moiety cleavable by cathepsin B at the $R_1 - X$ bond and which can be measured upon cleavage. Examples of X include AMC, AFC, AQ, HMC, PNP, PNA.

Salts of these compounds would include any salt of crystallization such as could be the case if X were AQ and this moiety formed the hydrochloride salt.

R_1 is an amino acid residue, having the L-configuration at the carbon designated α_1 above, that is not positively charged under the conditions of the assay. For example, the R_1 amino acid residue should generally not be positively charged within the pH range of from about 4 to about 8.

Referring to the structural formulae above, R_3 is a side chain of R_1 preferably containing from 2 to 8 atoms. Examples of R_3 include:

- CH_3 for R_1 is equal to Ala

$$-(CH_2)_3\text{-NH}\overset{\overset{\displaystyle NH}{\|}}{C}\text{-NH-NO}_2 \text{ for } R_1 \text{ is equal to } (NO_2)\text{ Arg}$$

$$-(CH_2)_3\text{-NH}\overset{\overset{\displaystyle O}{\|}}{C}\text{-NH}_2 \text{ for } R_1 \text{ is equal to Cit}$$

$$-(CH_2)_3\text{-NH}\overset{\overset{\displaystyle NH}{\|}}{C}\text{-NH-SO}_2C_6H_5\text{-CH}_3 \text{ for } R_1 \text{ is equal to (Ts) Arg}$$

$$-(CH_2)_4\text{-NH-}\overset{\overset{\displaystyle O}{\|}}{C}CF_3 \text{ for } R_1 \text{ is equal to (TFA) Lys}$$

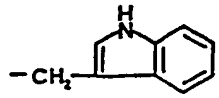
The L-configuration is necessary for binding cathepsin B. The purpose of excluding positively charged groups is to eliminate potential reactions with trypsin-like enzymes which would confound the assay or detection.

R_2 is a hydrophobic amino acid residue, preferably Phe, having the L-configuration at the α_2 carbon (Cα). The L-configuration is necessary for binding cathepsin B. The hydrophobic amino acid residue also enhances the binding of the substrate to cathepsin B.

Referring to the structural formula above, R_4 is a side chain to R_2 preferably having the formula:

$$-CH_2 - Y$$

where Y is a lower alkyl, phenyl, substituted phenyl, or indolyl group. By lower alkyl is meant 1 to 8 carbon atoms. Examples of R_4 include:



for R_2 is equal to Trp
- CH_2-C_6H_5 for R_2 is equal to Phe
- CH_2-CH-$(CH_3)_2$ for R_2 is equal to Leu
- CH-$(CH_3)_2$ for R_2 is equal to Val

The identity of Z is less critical than that of R_1 and R_2, but Z is useful in making small changes in affinity of the compound for cathepsin B. This Z group may be a simple amino protecting group, such as CBZ, BOC or Bz. Also, Z may be another blocked amino acid group, such as CBZ -(D or L configuration) - Ala. The Z group, in any event, should not interfere with the selective binding of cathepsin B to the R_1 and R_2 groups.

The synthesis of the peptide substrates can be effected by numerous generally accepted peptide synthetic methods. The following illustrates three synthetic strategies:

Method A: The group Z-R_2 is coupled to the R_1-X group by the DCC, mixed-anhydride or other typical coupling method.

Method B: The previously synthesized complete peptide Z-R_2-R_1 is coupled with the indicator group X using the DCC, mixed-anhydride or other acceptable coupling method.

Method C: Sequential coupling of the groups Z, R_2, R_1 is followed by the coupling of the indicator group X using the DCC, mixed-anhydride or other acceptable coupling method.

The general procedures for the majority of the compounds described in the examples are outlined below and follow the general *Method A*:

1. $R_{1\text{ or }2} + CBZ - Cl \xrightarrow[H_2O]{\text{base}} CBZ - R_{1\text{ or }2}$

2. $CBZ - R_1 + AMC \xrightarrow[\text{anhydride}]{\text{mixed}} CBZ - R_1 - AMC$

3. $CBZ - R_1 - AMC \xrightarrow[\text{or HBr/HOAc}]{H_2/Pd} H_2N - R_1 - AMC$

4. $CBZ - R_2 + H_2N - R_1 - AMC \xrightarrow[\text{anhydride}]{\text{mixed}} CBZ - R_2 - R_1 - AMC$

(Where R_1 and R_2 denote different amino acid residues in the general formula, and CBZ is used for Z and AMC is used for X).

The methods and compounds of the invention will now be further illustrated by the following non limiting examples:

Mixed anhydride refers to the type of coupling procedure. The details of reaction 1-4 are described below.

(1) *CBZ-Cl Reaction*: In some cases the CBZ-R derivatives were commercially available, in others they were prepared by the following method: The amino acid R (25 mmole), and 50 mmole sodium bicarbonate were dissolved into 50 ml of water in a 100-ml flask. Carbobenzyloxy -chloride (27.5 mmole) was added with stirring at ambient temperature in 5 aliquots over one hour. After the solution was stirred for an additional two hours, it was extracted with ether twice and then dripped into 100 ml of IM HCl. The product first precipitated as a semisolid and then solidified upon standing. The crude product was washed with water, dried and used without further purification. The average yield was 55%.

(2) *AMC - Coupling*: Into a 50-ml flask were added 11.4 mmole blocked R_1 - amino acid, 15 ml anhydrous THF, and 11.54 mmole TEA. After the compound dissolved, the solution was cooled on ice, and 11.4 mmole of isobutylchoroformate was added. The solution was stirred for 10 minutes on ice, and then a chilled solution of 11.4 mmole AMC in 21 ml of DMF was added. Stirring was continued on ice for one hour then at ambient temperature overnight. The reaction mixture was filtered and the THF removed from the filtrate on a rotary evaporator. The residue was added to a 125-ml separatory funnel along with 20 ml CH_2Cl_2 and 30 ml 10% aqueous HCl, and shaken vigourously. After extracting the organic phase with a second aqueous acid aliquot, the product precipitated in the organic phase. The aqueous phase was decanted and the precipitate collected by filtration and washed with ether. The product was dried and used without further purification. The average yield was 41%.

(3) *Deblocking*: The N-carbobenzyloxy blocking group was removed from Z-R_1 - AMC either by treatment with a hydrogen bromide/glacial acetic acid solution (HBr/HOAc), or catalytic hydrogenation. In two examples -t-BOC was used instead of CBZ, and deblocking was accomplished by formic acid treatment.

HBr/HOAc: Into a 500-ml flask were added 3.8 mmole of Z - R_1 - AMC and 70 ml of 33% HBr/HOAc. After the sample dissolved, the solution was stirred an additional 15 minutes. The reaction mixture was diluted with 500 ml ether and the resulting precipitate was collected by filtration under nitrogen. The product was resuspended three times in 100 ml ether and refiltered. The product was dried and used without further purification. The average yield was 98%.

Catalytic Hydrogenation: Into a 500-ml pressure flask were added 8.5 mmole Z-R_1-AMC, 4 g of palladium-polyethyleneimine beads (Pd-PEI) and 200 ml of methanol. The flask was pressurized with 20 psig (0.138 MPa gauge) hydrogen and shaken for six hours, then allowed to stand for an additional 18 hours. After the catalyst beads settled, the alcohol was decanted and the beads were washed twice with 50 ml of methanol. To aid the purification, the hydrochloride salt was prepared by adding 12.5 ml of 1 N aqueous HCl. The solvent was removed under vacuum and the residue redissolved and reevaporated twice with 50 ml denatured alcohol. The residue was triturated with denatured alcohol and allowed to stand overnight. The product, R_1-AMC was collected by filtration, washed with denatured alcohol, dried under vacuum, and used without further purification. The average yield was 78%.

Formic Acid: Into a 500-ml flask was added 1.75 mmole t-BOC-R_1-AMC and 30 ml of 97% formic acid. After the compound dissolved, the solution was stirred at ambient temperature for 3 hours. To this was added 250 ml of ether and 0.2 ml of concentrated HCl (to form the HCl salt). The white precipitate was filtered and washed with ether. After drying under vacuum, the product was used without further purification.

(4) *Peptide Coupling*: Into a 50-ml flask were added 3.2 mmole of R_1 - AMC · HBr (or HCl), 25 ml anhydrous DMF and 3.2 mmole TEA. Into a second 50-ml flask were added 3.2 mmole CBZ - R_2, 10 ml anhydrous DMF and 3.2 mmole TEA. After the compounds dissolved, both solutions were cooled on ice, and 3.2 mmole of isobutyl chloroformate was added to the CBZ - R_2 solution. This reaction mixture was stirred for 10 minutes on ice, and then the chilled R_1 - AMC solution was added to the CBZ -R_2 reaction mixture. Stirring was continued for one hour on ice and then overnight at room temperature. The reaction mixture was then dripped into 950 ml of 5% aqueous sodium bicarbonate with stirring. The precipitate was collected by filtration and washed three times with water. The average crude yield was 90%.

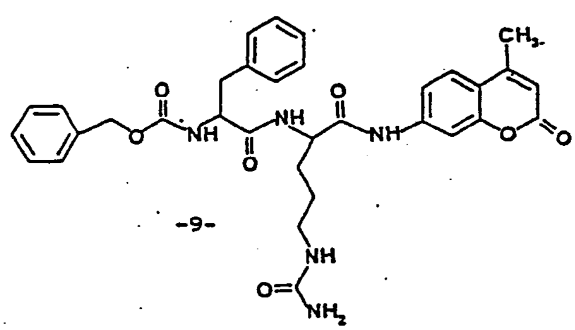
Purification: The final products were purified by the following crystallization and in some cases additional crystallizations and HPLC. The CBZ - R_2 - R_1 - AMC product (2.9 mmole) was dissolved into 35 ml of glacial acetic acid with stirring and gentle heating. To this was added 54 ml of acetonitrile and this solution was heated to reflux. The hot solution was filtered, and 80 ml of water was added to the filtrate in small aliquots with heating and stirring. A white precipitate formed upon cooling which was filtered and washed with water. The recrystallized product was dried under vacuum and the average crystallization yield was 80%. The overall yield and synthetic alterations are listed for each example below.

The chemical structures for the peptides discussed in this specification and appearing in the examples are given below:

Example

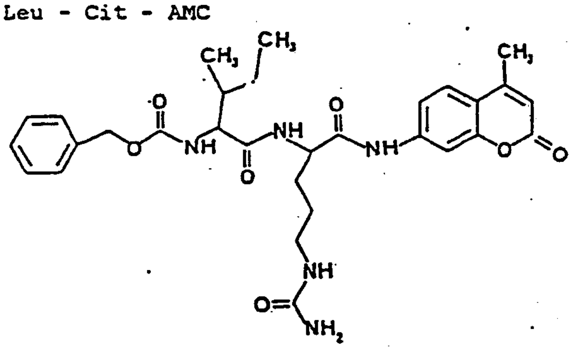
No. Compound or Substrate

1. CBZ - Phe - Cit - AMC

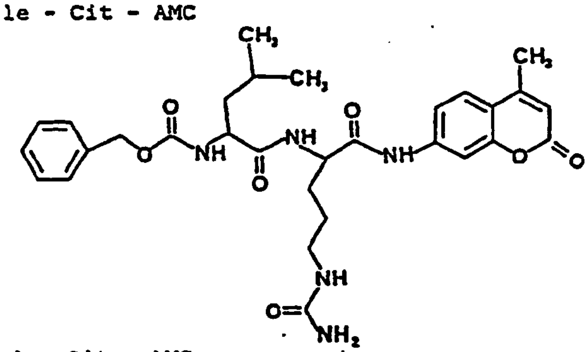


-9-

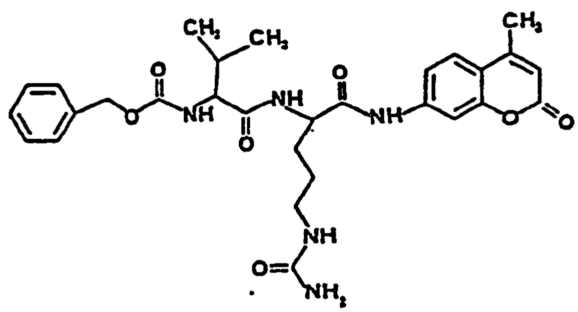
2. CBZ - Leu - Cit - AMC



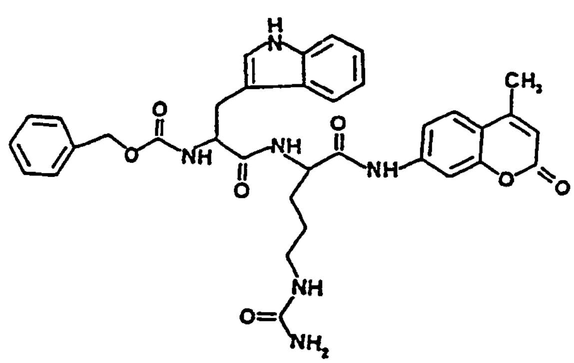
3. CBZ - Ile - Cit - AMC



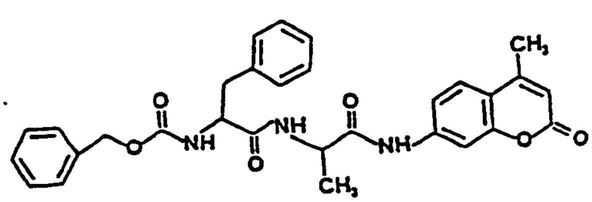
4. CBZ - Val - Cit - AMC



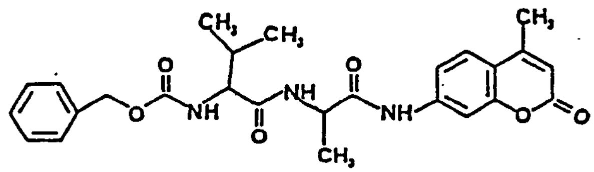
5. CBZ - Trp - Cit - AMC



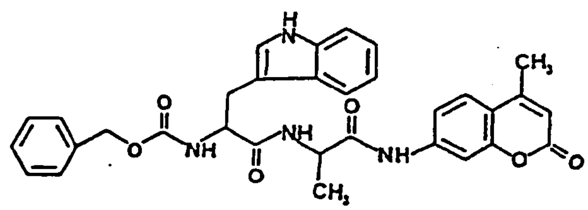
6. CBZ - Phe - Ala - AMC



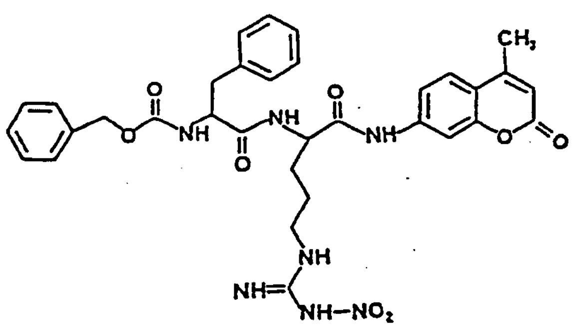
7. CBZ - Val - Ala - AMC



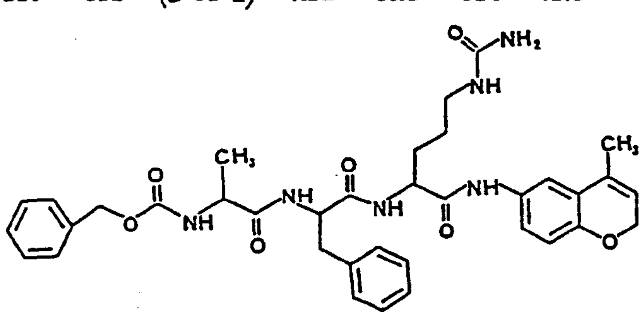
8. CBZ - Trp - Ala - AMC



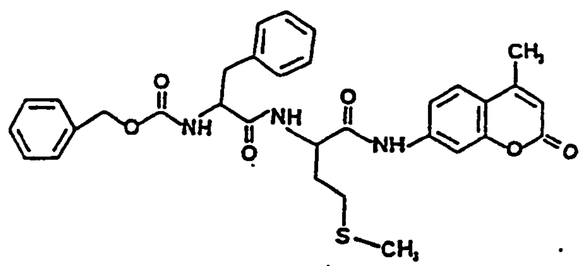
9. CBZ - Phe - (NO_2) Arg - AMC



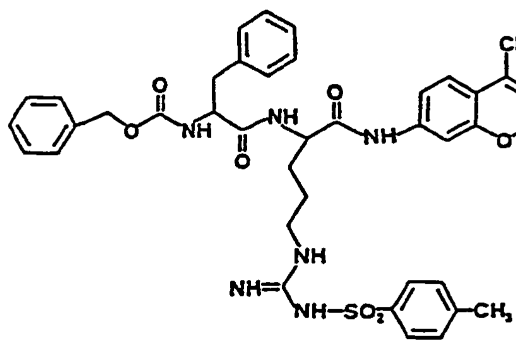
10, 11. CBZ - (D or L) - Ala - Phe - Cit - AMC



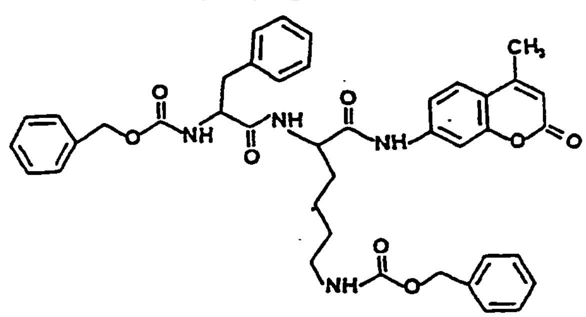
12. CBZ - Phe - Met - AMC



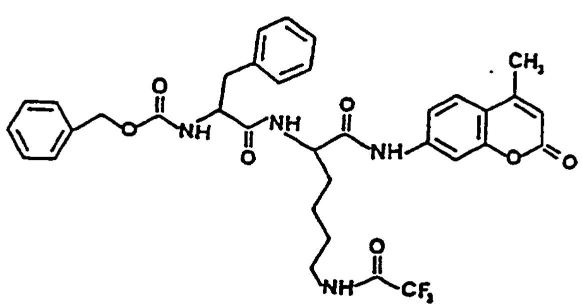
13. CBZ - Phe - (Ts) Arg - AMC



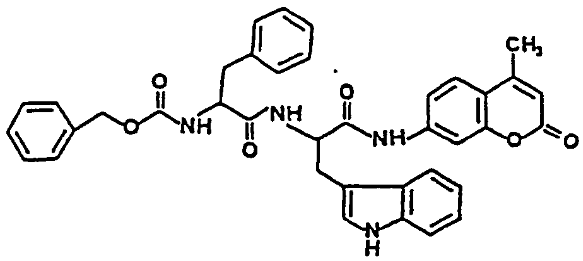
14. CBZ - Phe - (CBZ) Lys - AMC



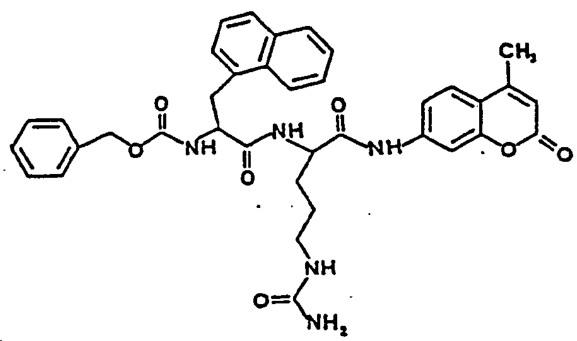
15. CBZ - Phe - (TFA) Lys - AMC



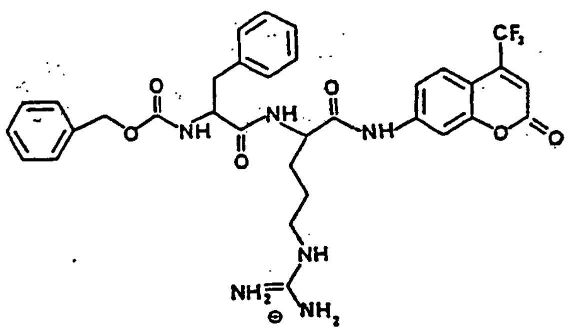
16. CBZ - Phe - Trp - AMC



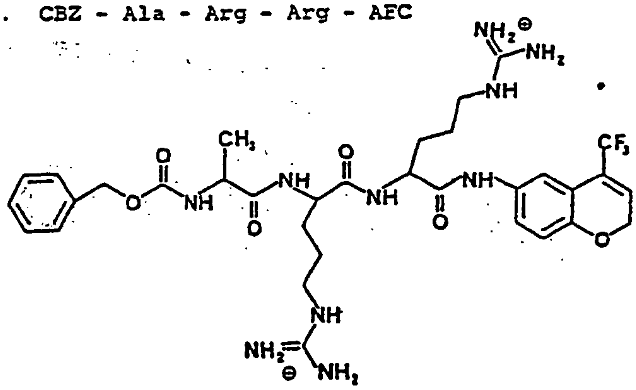
17. CBZ - Nala - Cit - AMC



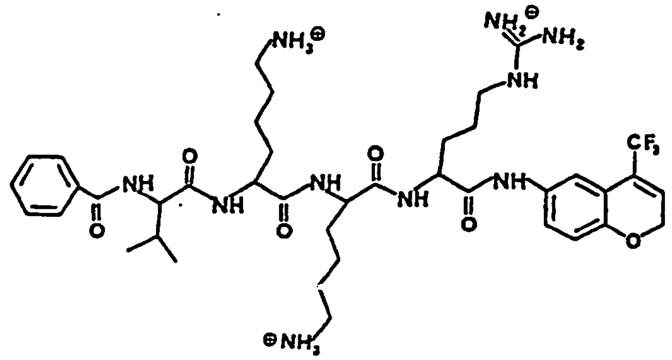
18. CBZ - Phe - Arg - AFC



19. CBZ - Ala - Arg - Arg - AFC



20. BZ - Val - Lys - Lys - Arg - AFC



Substrate Synthesis

Example 1: CBZ-L-Phe-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 17 ml HOAc, 74 ml CH_3CN, 140 ml H_2O per gram. The overall yield was 14%. *MP*=227-230°C. α_D^{25} (DMF) = −22.77°. *HPLC* (70% CH_3OH/30%H_2O) 9.6 min, 97.3%. *^{1}H-NMR: δ 1.30-1.55, c, 2p; δ 1.55-1.81, c, 2p; δ 2.40, s, 3p; δ 2.75, t, 1p; δ 2.90-310, c, 3p; δ 4.28-4.40, c, 1p; δ 4.40-4.51, c, 1p; δ 4.94, s, 2p; δ 5.43, s, 2p; δ 6.00, t, 1p; δ 6.28, s, 1p; δ 7.10-7.39, c, 10p; δ 7.48, d, 1p; δ 7.51, d, 1p; δ 7.73, d, 1p; δ 7.80, d, 1p; δ 8.35, d, 1p; δ 10.51, s, 1p. MS:* parent molecular ion = 614.

Elemental Analysis: calculated for $C_{33}H_{35}N_5O_7$, C 64.59%, H 5.75%, N 11.41%; found C 64.81%, H 5.77%, N 11.61%.

Example 2: CBZ-L-Leu-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 17 ml HOAc, 36 ml CH_3CN, 136 ml H_2O per gram. The overall yield was 19%. *MP*=196-198°C. $[\alpha]^{2}{}_{D}{}^{5}$ (DMF) = -25.79°. *HPLC*, (45% CH_3CN/55% H_2O) 9.8 min, 99.9%. *^{1}H-NMR*: δ 0.87, 2d, 6p; δ 1.44, t, 3p; δ 1.54-1.77, c, 4p; δ 2.39, s, 3p; δ 2.86-3.10, c, 2p; δ 4.10, q, 1p; δ 4.43, q, 1p; δ 5.04, s, 2p; δ 5.42, s, 2p; δ 5.98, t, 1p; δ 6.27, s, 1p; δ 7.24-7.53, c, 7p; δ 7.72, d, 1p; δ 7.78, d, 1p; δ 8.16, d, 1p; δ 10.46, s, 1p. *MS*: parent molecular ion = 580.

Elemental Analysis: calculated for $C_{30}H_{37}N_5O_7$, C 62.16% H 6.43%, N 12.08%; found C 62.59%, H 6.50%, H 12.06%.

Example 3: CBZ-L-Ile-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 20 ml HOAc, 34 ml CH_3CN, 54 ml H_2O per gram. The overall yield was 19%. *MP*=240-242°C. $[\alpha]_D^{25}$ (DMF) = -28.16°. HPLC (50%CH_3CN/50%H_2O) 7.1 min, 91.8%. *^{1}H-NMR*: δ 0.73-0.93, c, 6p; δ 1.00-1.80, c, 7p; δ 2.40, s, 3p; δ 2.86-3.12, c, 2p; δ 3.96, t, 1p; δ 4.43, q, 1p; δ 5.05, s, 2p; δ 5.43, s, 2p; δ 5.99, t, 1p; δ 6.27, s, 1p; δ 7.25-7.42, c, 6p; δ 7.49, d, 1p; δ 7.72, d, 1p; δ 7.78, s, 1p; δ 8.20, d, 1p; δ 10.46, s, 1p. *MS*: parent molecular ion = 580.

Elemental Analysis: calculated for $C_{30}H_{37}N_5O_7$, C 62.16%, H 6.43%, N 12.08%; found C 62.24%, H 6.36%, N 12.14%.

Example 4: CBZ-L-Val-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 24 ml HOAc, 75 ml CH_3CN, 128 ml H_2O per gram. The overall yield was 16%. *MP*=244-247°C. $[\alpha]_D^{25}$ (DMF) = -27.36°. *HPLC* (35%CH_3CN/65%H_2O) 17.1 min, 93.8%.

^{1}H-NMR: δ 0.73-1.0, c, 6p; δ 1.30-1.54, c, 2p; δ 1.54-1.83, c, 2p; δ 1.83-2.10, c, 1p; δ 2.40, s, 3p; δ 2.83-3.12, c, 2p; δ 3.94, t, 1p; δ 4.43, q, 1p; δ 5.05, s, 2p; δ 5.42, s, 2p; δ 5.98, t, 1p; δ 6.28, s, 1p; δ 7.20-7.43, c, 5p; δ 7.44-7.49, c, 2p; δ 7.72, d, 1p; δ 7.77, d, 1p; δ 8.19, d, 1p; δ 10.46, s, 1p.

MS: parent molecular ion = 566.

Elemental Analysis: calculated for $C_{29}H_{35}N_5O_7$, C 61.58%, H 6.24%, N 12.38%; found C 61.86%, H 6.27%, N 12.44%.

Example 5: CBZ-L-Trp-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 16 ml HOAc, 37 ml CH_3CN, 96 ml H_2O per gram. The overall yield was 17%. *MP*=235-238°C $[\alpha]_D^{25}$ (DMF) = -23.75°. *HPLC*, (45%CH_3CN/55%H_2O) 5.2 min, 95.6%.

^{1}H-NMR: δ 1.30-1.54, c, 2p; δ 1.54-183, c, 2p; δ 2.40, s, 3p; δ 2.82-3.40, c, 4p; δ 4.33-4.54, c, 2p; δ 4.96, s, 2p; δ 5.43, s, 2p; δ 5.99, t, 1p; δ 6.27, s, 1p; δ 6.95, t, 1p; δ 7.05, t, 1p; δ 7.10-7.45, c, 8p; δ 7.50, d, 1p; δ 7.65, d, 1p; δ 7.73, d, 1p; δ 7.80, d, 1p; δ 8.33, d, 1p; δ 10.49, s, 1p; δ 10.79, s, 1p.

MS: parent molecular ion = 653.

Elemental Analysis: calculated for $C_{35}H_{36}N_6O_7$, C 64.41% H 5.56%, N 12.88%; found C 64.46%, H 5.48%, H 12.94%.

Example 6: CBZ-L-Phe-L-Ala-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 18 ml HOAc, 27 ml CH_3CN, 29 ml H_2O per gram. The overall yield was 31%. *MP*=232-234°C. $[\alpha]_D^{25}$ (DMF) = -23.41°. *HPLC* (55%CH_3CN/45%H_2O) 6.8 min, 98.2%.

^{1}*H-NMR*: δ 1.36, d, 3p; δ 2.40, s, 3p; δ 2.74, t, 1p; δ 2.98-3.13, c, 1p; δ 4.33, c, 1p; δ 4.46, c, 1p; δ 4.94, s, 2p; δ 6.28, s, 1p; δ 7.12-7.40, c, 10p; δ 7.44-7.56, c, 2p; δ 7.74, d, 1p; δ 7.78, d, 1p; δ 8.40, d, 1p; δ 10.43, s, 1p.

MS: parent molecular ion = 528.

Elemental Analysis: calculated for $C_{30}H_{29}N_3O_6$, C 68.30%, H 5.54%, N 7.96%; found C 68.57%, H 5.68%, N 7.96%.

Example 7: CBZ-L-Val-L-Ala-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 100 ml HOAc, 104 ml CH_3CN, 67 ml H_2O per gram. The overall yield was 31%. *MP*=260-265°C. $[\alpha]_D^{25}$ (DMF = -34.51°. *HPLC* (45%CH_3CN/55%H_2O) 6.8 min, 98.7%.

^{1}*H-NMR*: δ 0.82-0.96, c, 6p; δ 1.33, d, 3p; δ 1.88-2.07, c, 1p; δ 2.40, s, 3p; δ 3.92, t, 1p; δ 4.44, c, 1p; δ 5.05, s, 2p; δ 6.28, s, 1p; δ 7.26-7.42, c, 5p; δ 7.45-7.52, c, 2p; δ 7.73, d, 1p; δ 7.76, d, 1p; δ 8.25, d, 1p; δ 10.39, s, 1p.

MS: parent molecular ion = 480.

Elemental Analysis: calculated for $C_{26}H_{29}N_3O_6$, C 65.12%, H 6.10%, N 8.76%; found C 65.59%, H 6.02%, N 8.79%.

Example 8: CBZ-L-Trp-L-Ala-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3, and recrystallization from 33 ml HOAc, 47 ml CH_3CN, 44 ml H_2O per gram. The overall yield was 17%. *MP*=248-253°C. $[\alpha]_D^{25}$ (DMF) = -16.55°. *HPLC* (45%CH_3CN/55%H_2O) 13.8 min, 93.4%.

^{1}*H-NMR*: δ 1.34, d, 3p; δ 2.40, s, 3p; δ 2.94, c, 1p; δ 3.08-3.22, c, 1p; δ 4.36, se, 1p; δ 4.45, t, 1p; δ 4.95, s, 2p; δ 6.26, s, 1p; δ 6.95, t, 1p; 7.05, t, 1p; δ 7.10-7.40, c, 8p; δ 7.50, d, 1p; δ 7.66, d, 1p; δ 7.72, d, 1p; δ 7.78, d, 1p; δ 8.36; d, 1p; δ 10.38, s, 1p; δ 10.79, s, 1p.

MS: parent molecular ion = 567.

Elemental Analysis: calculated for $C_{32}H_{30}N_4O_6$, C 67.83% H 5.34%, N 9.89%; found C 68.00%, H 5.42%, N 9.96%.

Example 9: CBZ-L-Phe-L-(NO_2)Arg-AMC was synthesized as detailed in steps 1-4 above using the HBr/HOAc deblocking in step 3, and recrystallization from 8 ml HOAc, 16 ml CH_3CN, 15 ml H_2O per gram. The overall yield was 20%. *MP*=150-160°C(dec). $[\alpha]_D^{25}$ (DMF) = -3.80°. *HPLC* (55%CH_3CN/45%H_2O) 8.8 min 95.9%.

^{1}*H-NMR*: δ 1.4-1.9, c, 4p; δ 2.4, s, 3p; δ 2.7-3.1, c, 2p; δ 3.19, c, 2p; δ 4.34, c, 1p; δ 4.45, c, 1p; δ 4.95, s, 2p; δ 6.28, s, 1p; δ 7.10-7.42, c, 13p; δ 7.49, d, 2p; δ 7.74, d, 1p; δ 7.78, d, 1p; δ 8.34, d, 1p; δ 10.49, s, 1p.

MS: parent molecular ion = 658.

Elemental Analysis: calculated for $C_{33}H_{35}N_4O_8$, C 60.27%, H 5.36%, N 14.91%; found C 60.03%, H 5.41%, N 15.09%.

Example 10: CBZ-L-Ala-L-Phe-L-Cit-AMC was synthesized from the CBZ-L-Phe-L-Cit-AMC, (Example 1) by deblocking with HBr/HOAc as in step 3 above, followed by peptide coupling of that reaction product with CBZ-L-Ala as in step 4 above. The final product was recrystallized from 34 ml POAc, 44 ml CH_3CN, 273 ml H_2O per gram, and further purified for enzymatic analysis by *HPLC* (Prep., 45%CH_3CN/55%H_2O, 20 min). The overall crude yield was 40%. *MP*=236-240°C. $[\alpha]_D^{25}$ (DMF = -39.91°. *HPLC* (60%CH_3CN/40%H_2O) 6.5 min, 92.1%.

^{1}*H-NMR*: δ 1.14, d, 3p; δ 1.28-1.54, c, 2p; δ 1.54-1.83, c, 2p; δ 2.42, s, 3p; δ 2.73-3.14, c, 4p; δ 4.02, t, 1p; δ 4.44, q, 1p; δ 4.56, c, 1p; δ 5.00, q, 2p; δ 5.43, s, 2p; δ 5.99, t, 1p; δ 6.28, s, 1p; δ 7.08-7.40, c, 10p; δ 7.45, d, 1p; δ 7.53, d, 1p; δ 7.74, d, 1p; δ 7.79, d, 1p; δ 7.90, d, 1p; δ 8.28, d, 1p; δ 10.44, s, 1p.

MS: parent molecular ion = 685.

Elemental Analysis: calculated for $C_{36}H_{40}N_6O_8$, C 63.15%, H 5.89%, N 12.27%; found C 63.10%, H 5.91%, N 12.15%.

Example 11: CBZ-D-Ala-L-Phe-L-Cit-AMC was synthesized similar to Example 10 using CBZ-D-Ala and L-Phe-L-Cit-AMC in the final peptide coupling. The final product was recrystallized from 13 ml HOAc, 36 ml CH_3CN, 173 ml H_2O per gram, and further purified for enzymatic analysis by *HPLC* (Prep., 45%CH_3CN/55%H_2O, 20 min). The overall yield was 34%. *MP*=194-196°C. $[\alpha]_D^{25}$ (DMF) = -17.89°. *HPLC*, (60%CH_3CN/40%H_2O) 6.5 min, 88.4%.

^{1}*H-NMR*: δ 0.98, d, 3p; δ 1.28-1.56, c, 2p; δ 1.56-1.88, c, 2p; δ 2.41, s, 3p; δ 2.78-3.20, c, 4p; δ 4.03, t, 1p; δ 4.44, c, 1p; δ 4.58, c, 1p; δ 4.95, q, 2p; δ 5.42, s, 2p; δ 6.00, t, 1p; δ 6.28, s, 1p; δ 7.10-7.38, c, 10p; δ 7.44, d, 1p; δ 7.54, d, 1p; δ 7.73, d, 1p; δ 7.80, d, 1p; δ 8.19, d, 1p; δ 8.24, d, 1p; δ 10.32, s, 1p.

MS: parent molecular ion = 685.

Elemental Analysis: calculated for $C_{36}H_{40}N_6O_8$, C 63.15% H 5.89%, N 12.27%; found C 62.97%, H 5.91%, N 12.20%.

Example 12: CBZ-L-Phe-L-Met-AMC was synthesized as detailed in steps 1-4 above using the HBr/HOAC deblocking in step 3 and recrystallization from 3 ml HOAc, 10 ml CH_3CN, 4 ml H_2O per gram, and further purified by *HPLC* (Prep., 60%CH_3CN/40%H_2O, 15 min) for enzymatic assay. The overall yield was 16%. *MP*=202-209°C. $[\alpha]_D^{25}$ (DMF) = -3.79°. *HPLC*, (45%CH_3CN/55%H_2O) 17.3 min, 85.2%.

^{1}*H-NMR*: δ 1.85-2.20, c, 2p; δ 2.07, s, 3p; δ 2.35-2.62, c, 2p; δ 2.42, s, 3p; δ 2.76, t, 2p; δ 4.34, c, 1p; δ 4.53, c, 1p; δ 4.96, s, 2p; δ 6.28, s, 1p; δ 7.14-7.39, c, 10p; δ 7.50, d, 2p; δ 7.74, d, 1p; δ 7.78, d, 1p; δ 8.36, d, 1p; δ 10.48, s, 1p.

MS: parent molecular ion = 588.

Elemental Analysis: calculated for $C_{32}H_{33}N_3O_6S$, C 65.40% H 5.66%, N 7.15%, S 5.46, found C 65.76%, H 5.77%, N 7.09, S 5.45%.

Example 13: CBZ-L-Phe-L-(Ts)Arg-AMC was synthesized as detailed in steps 2-4 starting with the α-t-BOC(Ts)Arg instead of the α-CBZ derivative in step 2. The α-t-BOC group was removed by formic acid as described above with a 17% yield. The overall crude yield was 9.2% and this material was further purified by *HPLC* (Prep., 75%CH_3OH/25%H_2O, 20 min). *MP*=118-125°C. $[\alpha]_D^{25}$ + 7.0°. *HPLC*, (65%CH_3CN/35%H_2O) 11.3 min, 90.9%.

^{1}*H-NMR*: δ 1.29-1.58, c, 2p; δ 1.58-1.86, c, 2p; δ 2.28, s, 3p; δ 2.40, s, 3p; δ 2.74, t, 1p; δ 2.92-3.20, c, 3p; δ 4.24-4.52, d, 2p; δ 4.95, s, 2p; δ 6.28, s, 1p; δ 6.43-7.11, c, 3p; δ 7.11-7.44, c, 14p; δ 7.62, d, 2p; δ 7.75, d, 1p; δ 7.78, s, 1p; δ 8.34, c, 1p; δ 10.52, s, 1p.

MS: parent molecular ion = 767.

Elemental Analysis: calculated for $C_{40}H_{42}N_6O_8S$, C 62.65% H 5.52%, N 10.96%, S 4.18; found C 62.04%, H 5.66%, N 10.90, S 4.47%.

Example 14: CBZ-L-Phe-L-(CBZ)Lys-AMC was synthesized as detailed in steps 2-4 starting with the α-t-BOC(CBZ)Lys instead of the α-CBZ derivative in step 2. The α-t-BOC group was removed by formic acid as described above with a 83% yield. Recrystallization was from 20 ml acetic acid, 56 ml CH_3CN, 127 ml H_2O per gram. The overal yield was 39%. *MP*=170-173°C. $[\alpha]_D^{25}$ (DMF) = -4.28°. *HPLC*, (45%CH_3CN/55%H_2O) 15.3 min, 97.5%.

^{1}*H-NMR*: δ 1.20-1.54, c, 4p; δ 1.54-1.82, c, 2p; δ 2.40, s, 3p; δ 2.76, t, 2p; δ 2.88-3.09, c, 2p; δ 4.28-4.48, c, 2p; δ 4.96, d, 4p; δ 6.27, s, 1p; δ 7.10-7.43, c, 17p; δ 7.49, t, 1p; δ 7.73, d, 1p; δ 7.77, s, 1p; δ 8.29, d, 1p; δ 10.47, s, 1p.

MS: parent molecular ion = 719.

Elemental Analysis: calculated for $C_{41}H_{42}N_4O_8$, C 68.51% H 5.89%, N 7.79%; found C 68.23%, H 5.91%, N 7.69%.

Example 15: CBZ-L-Phe-L-(TFA)Lys-AMC was synthesized as detailed in steps 1-4 using the catalytic hydrogenation deblocking in step 3 and recrystallization from 28 ml CH_3 CN per gram. The overall yield was 9%. *MP*=208-210°C. $[\alpha]_D^{25}$ (DMF) = -8.15°. *HPLC*, (45%CH_3CN/55%H_2O) 15.8 min, 80.4%.

^{1}*H-NMR*: δ 1.20-1.45, c, 2p; δ 1.45-1.61, c, 2p; δ 1.61-1.85, c, 2p; δ 2.42, s, 3p; δ 2.75-3.06, c, 2p; δ 3.18, c, 2p; δ 4.28, se, 1p; δ 4.48, q, 1p; δ 4.95, s, 2p; δ 6.28, s, 1p; δ 7.14-7.39, c, 10p; δ 7.44-7.54, c, 2p; δ 7.74, d, 1p; δ 7.78, d, 1p; δ 8.31, d, 1p; δ 9.40, c, 1p; δ 10.48, s, 1p.

MS: parent molecular ion = 681.

Elemental Analysis: calculated for $C_{35}H_{35}N_4O_7F_3$, C 61.76% H 5.18%, N 8.23%, F 8.37%; found C 62.28%, H 5.38%, N 8.30, F 8.06%.

Example 16: CBZ-L-Phe-L-Trp-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3 and recrystallization from 11 ml HOAc, 11 ml CH_3CN, 4 ml and H_2O per gram. The overal yield was 31%. *MP*=165-167°C. $[\alpha]_D^{25}$ (DMF) = +51.89°. *HPLC*, (45%CH_3CN/55%H_2O) 16.2 min, 99.4%.

^{1}*H-NMR*: δ 2.40, s, 3p; δ 2.74, t, 2p; δ 2.83-3.30, c, 2p; δ 4.33, qn, 1p; δ 4.76, q, 1p; δ 4.97, s, 2p; δ 6.28, s, 1p; δ 6.97, t, 1p; δ 7.07, t, 1p; δ 7.12-7.38, c, 13p; δ 7.48, d, 1p; δ 7.63, d, 1p; δ 7.72, d, 1p; δ 7.76, s, 1p; δ 8.38, d, 1p; δ 10.52, s, 1p; δ 10.86, s, 1p.

MS: parent molecular ion = 643.

Elemental Analysis: calculated for $C_{38}H_{34}N_4O_6$, C 71.01% H 5.33%, N 8.72%; found C 71.21%, H 5.40%, N 8.72%.

Example 17: CBZ-L-Nala-L-Cit-AMC was synthesized as detailed in steps 1-4 above using the catalytic hydrogenation deblocking in step 3 and recrystallization from 40 ml HOAc, 86 ml CH_3CN, 92 ml and H_2O per gram. The overal yield was 13%. *MP*=225-227°C. $[\alpha]_D^{25}$ (DMF)= −20.3°. *HPLC*, (60%CH_3CN/40%H_2O) 8.0 min, 99.9%.

^{1}*H-NMR*: δ 1.80-1.56, c, 2p; δ 1.56-1.85, c, 2p; δ 2.40, s, 3p; δ 2.84-3.22, c, 4p; δ 4.38-4.57, c, 2p; δ 4.91, s, 2p; δ 5.43, s, 2p; δ 6.00, t, 1p; δ 6.28, s, 1p; δ 7.07-7.65, c, 12p; δ 7.74, d, 1p; δ 7.78, d, 1p; δ 7.92, d, 1p; δ 8.22, d, 1p; δ 8.34, d, 1p; δ 10.50, s, 1p.

MS: parent molecular ion = 664.

Elemental Analysis: calculated for $C_{37}H_{37}N_5O_7$, C 64.59% H 5.75%, N 11.41%; found C 64.68%, H 5.87, N 11.40%.

Assay

The activity of cathepsin B is detected by monitoring the rate of release of indicator group X. The indicator group may be a moiety whose fluorescence or absorbance changes upon cleavage. Any of the known indicators such as AMC, HMC, AFC, AQ or PNA may be used. Alternatively, a radioactively labeled indicator may be employed.

The assay procedures for this enyme, cathepsin B, follow the standard procedures and general principles adapted for enzymes (as described in A. J. Barrett, *op. cit.*; W. P. Jencks (1969) *Catalysis in Chemistry and Enzymology*, McGraw Hill, New York). A steady-state kinetic condition is set up using appropriate enzyme/substrate concentration ratios (the substrate concentration and its Michaelis constant greatly exceed the enzyme concentration) in order to obtain accurate rate information in the shortest laboratory time scale.

The assay for cathepsin B activity is performed by adding a small quantity of a body fluid sample (1-50 microliters, μl) to an aqueous solution having a pH range at which cathepsin B is active, usually pH 5-6.5, preferably pH5.5, containing EDTA and a thiol activator (biological antioxidant or other suitable reducing agent) such as cysteine of dithiothreitol. After a short activation period such as 1-2 minutes the substrate, dissolved in a compatible solvent such as methanol, ethanol, acetonitrile or DMF, is added in a small volume (20 μl) such that the concentration of the substrate is substantially greater than the enzyme concentration (preferably at least 100 fold). The temperature is controlled to a set point, preferably in the range of ambient to about 37°C. After the sample and substrate are mixed, the concentration of cleaved indicator can be measured with respect to time in a spectrophotometer, spectrofluorometer of scintillation counter or other known means which are well-known art and form no part of this invention.

A standard curve of known free indicator concentrations is developed so as to give a scale of comparison for unknown samples. The calculations would be as follows for a fluorescent indicator:

Equation 1: A · B = C

specifically,

$$(\text{A fluorescence units/min})\ (\text{B}\ \frac{\mu\text{M indicator}}{\text{fluorescence units}}\ ) = \ (\text{C}\ \mu\text{M indicator/min.})$$

where A is obtained from the measurement with the sample, B is obtained from the standard curve, and C is the result. The term "μ*M*" refers to micromolar (μmoles/liter), "ml" to milliliters, and min to minutes. Calculations would be made in a similar manner when other indicators are used. Information from this calculation provides a rate in terms of the concentration of the free indicator group X, and its change with time. This rate can be directly related to the rate of substrate cleavage by the enzyme, and thus, the amount of enzyme activity can be inferred.

Assay Examples

Purified cathepsin B was prepared from pig liver and neoplastic human liver using standard salting out procedures. The assay was performed using a 0.1 *M* Bis-Tris, acetate of phosphate buffer having pH 5.5 and containing 1m*M* EDTA. Cysteine (10m*M*) was used as the thiol activator, and the buffer containing the activator was freshly made each day. A small volume (5μl) of sample containing cathepsin B was mixed with 585 μl of 37°C fresh buffer containing thiol activator in a 5x5 mm fluorimeter cuvette and incubated at 37°C for 2 minutes. Then 20μl of substrate in DMF at a concentration ranging from 0.05 mM to 3 mM was added with mixing. The increase in fluorescence was monitored with time at 37°C for several minutes using a Perkin Elmer LS-5 spectrofluorimeter. For AMC, the excitation wavelength was 350 nm, the emission wavelength 460 nm, and for AFC the excitation wavelength was 385 nm, the emission wavelength 490 nm. The fluorimeter settings used were: slit widths, 5 and 5mm, filter response, 1, and the scale factor was set at 0-1.0. The results of the enzymatic interaction with the substrate examples 1-17 and the commercial standards CBZ-Phe-Arg-AFC (Example 18), CBZ-Ala-Arg-Arg-AFC (Example 19) and Bz-Val-Lys-Lys-Arg-AFC (Example 20) are listed in Table 1.

TABLE 1

Ex No.	Compound	V_{max}/K_m cathepsin B	Trypsin	Rel. Order of Value[3]
1	CBZ-L-Phe-L-Cit-AMC[1]	0.296	NR[2]	2
2	CBZ-L-Leu-L-Cit-AMC	0.116	NR	6
3	CBZ-L-Ile-L-Cit-AMC	0.126	NR	5
4	CBZ-L-Val-L-Cit-AMC	0.169	NR	3
5	CBZ-L-Trp-L-Cit-AMC	0.148	NR	4
6	CBZ-L-Phe-L-Ala-AMC	0.034	NR	13
7	CBZ-L-Val-L-Ala-AMC	0.018	NR	14
8	CBZ-L-Trp-L-Ala-AMC	0.069	NR	8
9	CBZ-L-Phe-L-(NO_2)Arg-AMC	1.025	NR	1
10	CBZ-L-Ala-L-Phe-L-Cit-AMC	0.105	NR	7
11	CBZ-D-Ala-L-Phe-L-Cit-AMC	0.062	NR	15
12	CBZ-L-Phe-L-Met-AMC[4]	0.614	NR	12
13	CBZ-L-Phe-L-(Ts)Arg-AMC[4]	1.575	NR	9

1 AMC has a sensitivity of 0.049 μM/fluorescense unit and was used as B in Equation 1 for these calculations.
2 NR - no reaction.
3 The order is based upon the rate of reaction with cathepsin B (the higher the rate the lower the order) and the rate of reaction with trypsin (the higher the rate the higher the order).
4 The relative value for this compound is reduced due to low solubility and low maximum rate.

TABLE 1 (continued)

Ex No.	Compound	V_{max}/K_m cathepsin B	Trypsin	Rel. Order of Value[1]
14	CBZ-L-Phe-L-(Z)Lys-AMC[4]	0.182	NR	11
15	CBZ-L-Phe-L-(TFA)Lys-AMC[4]	0.457	NR	10
16	CBZ-L-Phe-L-Trp-AMC	0.01	NR	17
17	CBZ-L-Nala-L-Cit-AMC	0.01	NR	16
18	CBZ-Phe-Arg-AFC[5]	0.007	0.003	12
19	CBZ-Ala-Arg-Arg-AFC[5]	0.003	0.058	14
20	BZ-Val-Lys-Lys-Arg-AFC[5]	0.017	0.077	13

From these data it is clear that substrates 1-17 (Table 1) of the present invention have a much higher degree of selectivity and sensitivity for detecting cathepsin B in the presence of trypsin than the currently used peptides, such as the substrates 18-20 in Table 1 or similar peptides having a positively charged group at the R_1 position (attached to X). Since the tryptic enzymes are normally found in human blood, these enzymes interfere with cathepsin B measurement. The peptide substrates of the invention avoid or reduce the interference problems caused by trypsin-like enzymes, and increase the sensitivity for cathepsin B by 60-350 times that of the peptides of Examples 18-20.

In accordance with another embodiment of the invention,.the activity of cathespin B can be measured on a qualitative or semi-quantitative basis by contacting a prepared sample of the mammalian fluid with a suitable substrate of the invention. Typical clinical laboratory practices for obtaining such analyses might include, for example, cytology analysis in which the cell, tissue or organ sample is contacted with the substrate and other reagents for selective staining purposes. Still other typical clinical analyses include a test strip in which the substrate is incorporated into a suitable, relatively inert carrier which may be cellulosic or nonwoven material, or the combination thereof. When the sample is contacted with a test strip. the sample is absorbed into the test strip so as to result in a color change for a relatively quick analysis.

5 AFC has a sensitivity of 0.009 μM/fluorescence unit and was used as B in Equation 1 for these calculations.

CLAIMS

1. A method of selectively assaying for the activity of cathepsin B in a material to be tested which may

contain trypsin and trypsin-like enzymes, the steps comprising:

(a) mixing a sample of said material with a substrate of formula I

$$Z - R_2 - R_1 - X \qquad (I)$$

(wherein:

X is an indicator moiety released by cleavage of the $R_1 - X$ bond by cathepsin B and which is detectable upon cleavage;

R_1 is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the assay;

R_2 is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the R_1 and R_2 groups) of an acid salt thereof,

said mixing being carried out in an aqueous medium having a pH within the range at which cathepsin B is active and using a quantity of said substrate substantially greater than that of cathepsin B and in sufficient concentration for cleaved X-groups to be detectable; and

(b) measuring the rate of cleavage of the X-group from said substrate.

2. A method of detecting the activity of cathepsin B in a material to be tested which may contain trypsin and trypsin-like enzymes, and which is adapted to selectively assay for cathepsin B, the steps comprising:

(a) contacting a sample of said material with a substrate of formula I

$$Z - R_2 - R_1 - X \qquad (I)$$

(wherein:

X is an indicator moiety released by cleavage of the $R_1 - X$ bond by cathepsin B and which is detectable upon cleavage;

R_1 is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group and which is not positively charged within the pH range for the detection method;

R_2 is a hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group that does not interfere with the selective binding of cathepsin B to the R_1 and R_2 groups) or an acid salt thereof,

said contacting being carried out under conditions at which cathepsin B is active and at concentrations at which said substrate is present in amounts substantially greater than that of cathepsin B; and

(b) determining the production of X cleaved from said substrate.

3. The method of either of claims 1 and 2 wherein X is a cleavable moiety whose fluorescence emission changes upon cleavage.

4. The method of any one of claims 1 to 3 wherein X is a cleavable moiety whose visible or ultraviolet spectrum changes upon cleavage.

5. The method of either of claims 1 and 2 wherein X is a 7-amino-4-methylcoumarin, 7-hydroxy-4-methylcoumarin, 7-amino-4-trifloromethylcoumarin, 6-amino-quinoline, p-nitrophenol or p-nitroaniline group.

6. The method of any one of claims 1 to 5 wherein R_1 is a citrullyl group.

7. The method of any one of claims 1 to 5 wherein R_1 is a nitroarginyl group.

8. The method of any one of claims 1 to 7 wherein R_2 is a phenylalanyl group.

9. The method of any one of claims 1 to 7 wherein R_2 is a leucyl group.

10. The method of any one of claims 1 to 7 wherein R_2 is a tryptophyl group.

11. The method of any one of claims 1 to 7 wherein R_2 is an isoleucyl group.

12. The method of any one of claims 1 to 7 wherein R_2 is a valyl group.

13. The method of any one of claims 1 to 5 wherein R_2 is a phenylalanyl group and R_1 is a citrullyl group.

14. The method of any one of claims 1 to 5 wherein R_2 is a phenylalanyl group and R_1 is a nitroarginyl group.

15. The method of any one of claims 1 to 5 wherein R_1 is a citrullyl group and R_2 is a valyl group.

16. The method of any one of claims 1 to 5 wherein R_1 is a citrullyl group and R_2 is a tryptophyl group.

17. The method of any one of claims 1 to 5 wherein R_1 is a citrullyl group and R_2 is an isoleucyl group.

18. The method of any one of claims 1 to 17 wherein Z is a carbobenzyloxy, benzoyl or t-butyloxycarbonyl group.

19. The method of any one of claims 1 to 18 wherein said material is a mammalian body fluid.

20. A method substantially as herein before described for determining cathepsin B activity in fluids using a compound of formula I (as defined in claim 1 or 2) or an acid salt thereof.

21. Compounds of formula I

$$Z - R_2 - R_1 - X \qquad (I)$$

(wherein:

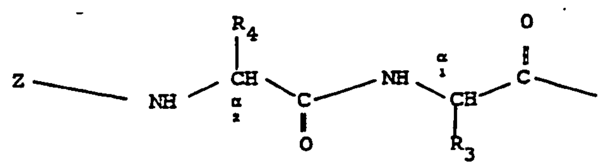
X is an indicator moiety cleavable from R_1;

R_1 is an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group, and that is not positively charged;

R_2 is an hydrophobic amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group; and

Z is an amino blocking group) and acid salts thereof.

22. The compounds of claim 21 wherein R_1 is a citrullyl group.
23. The compounds of claim 21 wherein R_1 is a nitroarginyl group.
24. The compounds of any one of claims 21 to 23 wherein R_2 is a phenylalanyl group.
25. The compounds of any one of claims 21 to 23 wherein R_2 is a leucyl group.
26. The compounds of any one of claims 21 to 23 wherein R_2 is a tryptophyl group.
27. The compounds of any one of claims 21 to 23 wherein R_2 is an isoleucyl group.
28. The compounds of any one of claims 21 to 23 wherein R_2 is a valyl group.
29. The compounds of claim 21 wherein R_2 is a phenylalanyl group and R_1 is a citrullyl group.
30. The compounds of claim 21 wherein R_2 is a phenylalanyl group and R_1 is a nitroarginyl group.
31. The compounds of claim 21 wherein R_1 is a citrullyl group and R_2 is a valyl group.
32. The compounds of claim 21 wherein R_1 is a citrullyl group and R_2 is a tryptophyl group.
33. The compounds of claim 21 wherein R_1 is a citrullyl group and R_2 is an isoleucyl group.
34. Compounds of formula II


(II)

(wherein:

X and Z are as defined in claim 21;

R_3 is the 2 to 8 atom containing side chain of an amino acid residue which has the L-configuration at the carbon alpha to the carbonyl group, and that is not positively charged; and

R_4 is the side chain of an hydrophobic amino acid residue which has the L-configuration at the carbon atom alpha to the carbonyl atom and R_4 has the following formula:

$$-CH_2-Y$$

wherein Y is selected from lower alkyl, phenyl, substituted phenyl and indole groups) and acid salts thereof.

35. The compounds of claim 34 wherein R_3 is

$$-CH_2-CH_2-CH_2-NH-\overset{\overset{\displaystyle O}{\|}}{C}-NH_2$$

36. The compounds of claim 34 wherein R_3 is

$$-CH_2-CH_2-CH_2-NH-\overset{\overset{\displaystyle NH}{\|}}{C}-NH-NO_2$$

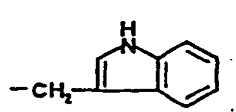
37. The compounds of any one of claims 34 to 36 wherein R_4 is

$$-CH_2-C_6H_5$$

38. The compounds of any one of claims 34 to 36 wherein R_4 is

$$-CH_2-CH(CH_3)_2$$

39. The compounds of any one of claims 34 to 36 wherein R_4 is



40. The compounds of any one of claims 34 to 46 wherein R_4 is

$-CH-(CH_3)_2$.

41. The compounds of any one of claims 34 to 36 wherein R_4 is

$$-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_2-CH_3$$

42. The compounds of claim 34 wherein R_4 is $-CH_2-C_6H_5$ and R_3 is

$$CH_2-CH_2-CH_2-NH-\overset{\displaystyle O}{\overset{\|}{C}}-NH_2$$

43. The compounds of claim 34 wherein R_4 is $-CH_2-C_6H_5$ and R_3 is

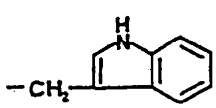
$$CH_2-CH_2-CH_2-NH-\overset{\displaystyle NH}{\overset{\|}{C}}-NH-NO_2$$

44. The compounds of claim 34 wherein R_3 is

$$-CH_2-CH_2-CH_2-NH-\underset{\displaystyle O}{\underset{\|}{C}}-NH_2 \quad \text{and } R_4 \text{ is} \quad -\underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_3$$

45. The compounds of claim 34 wherein R_3 is

$$-CH_2-CH_2-CH_2-NH-\overset{\displaystyle O}{\overset{\|}{C}}-NH_2 \quad \text{and } R_4 \text{ is}$$



46. The compounds of claim 34 wherein R_3 is

$$-CH_2-CH_2-CH_2-NH-\overset{\displaystyle O}{\overset{\|}{C}}-NH_2 \quad \text{and } R_4 \text{ is} \quad \underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_2-CH_3$$

47. The compounds of any one of claims 21 to 46 wherein X is a cleavable moiety whose fluorescense changes upon cleavage.

48. The compounds of any one of claims 21 to 47 wherein X is a cleavable moiety whose visible or ultra-violet spectrum changes upon cleavage.

49. The compounds of any one of claims 21 to 48 wherein X is a 7-amino-4-methylcoumarin, 7-hydroxy-4-methylcoumarin, 7-amino-4-trifloromethylcoumarin, 6-aminoquinoline, p-nitrophenol or p-nitroaniline group.

50. The compounds of any one of claims 21 to 49 wherein Z is a carbobenzyloxy, benzoyl or t-butyloxycarbonyl group.

51. Compounds as claimed in any one of claims 21 to 50 substantially as hereinbefore described in any one of Examples 1 to 17.

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